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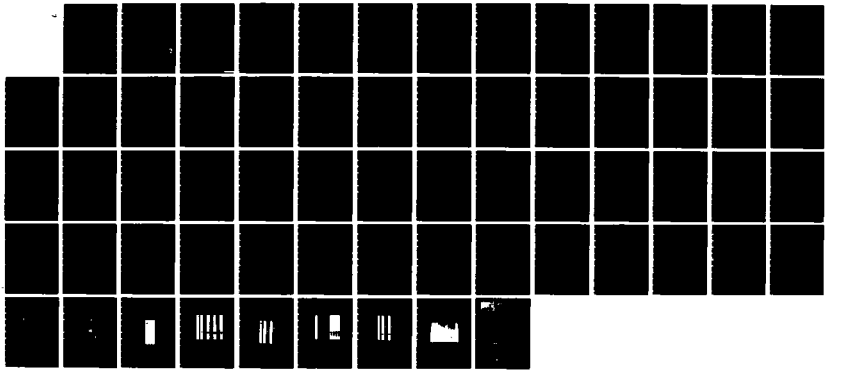

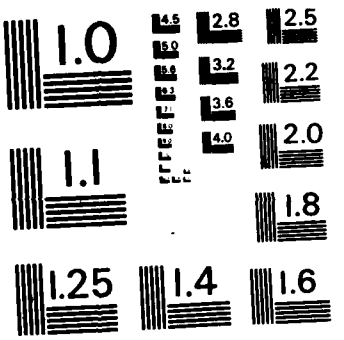

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EFFECTS OF TRYPANOCIDAL DRUGS ON THE FUNCTION OF TRYPANOSOMES

Annual Report

by

George C. Hill

February 1982

Supported by

US Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

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Colorado State University
Fort Collins, Colorado 80523

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During the past 12 months, we have continued our research on the biochemistry of African trypanosomes. We have characterized the in vitro system for growing cultured infective trypomastigotes of African trypanosomes and evaluated the activity of trypanocidal drugs in this system. Suramin and berenil have been shown to be extremely effective. In addition, we tested allopurinol and allopurinol ribonucleoside on T. rhodesiense cultured infective trypomastigotes. Inhibitory action was observed in vitro at 10-20 ug/ml. Efforts are now under way to analyze the acid extracted cells for any aminopurinol nucleotides by HPLC technique.

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Further attempts were made to isolate a mitochondrial ribosome from T. brucei. While it was possible to clearly identify 9S to 12S RNA, the presumable mitochondrial RNAs of trypanosomes, it was not possible to identify ribosomal subunits where 9S RNA was separated from 12S RNA. Given the extremely small amount of material that we have been able to obtain from mitochondrial lysates, we do not believe further pursuit of this problem is warranted at this time.

One and two-dimensional electrophoresis of mitochondria from T. rhodesiense determined that the main differences between the two types of mitochondria is quantitative and not qualitative. Most of the same proteins are present in both mitochondria but in apparent varying amounts. Using a monoclonal antibody specific for subunit II of cytochrome oxidase (COX II), a protein was identified in the one-dimensional system. Whether this protein is COX II or a closely related protein remains to be determined.

Efforts have been made to identify a restriction enzyme fragment of the maxicircle of kinetoplast DNA which hybridizes with a probe which is the cloned gene for cytochrome oxidase subunit II from yeast. This maxicircle fragment has been identified as a 2.8 kbp Taq 1 fragment. These experiments allowed us to develop recombinant DNA technology in our laboratory which will be important with regards to new experiments proposed to identify developmentally regulated clones in procyclic and bloodstream trypomastigotes of T. brucei.

Using 0.08% or 0.2% deoxycholate, it has been possible to solubilize the α-glycerophosphate oxidase from the mitochondrial preparation of T. gambiense. Efforts are now under way to further purify this enzyme and to identify the protein band(s) which corresponds to the α-glycerophosphate oxidase activity.

Our efforts during the next year will concentrate on the identification of developmentally regulated clones of bloodstream and procyclic trypomastigotes of T. brucei, further purification of the α-glycerophosphate oxidase system from T. brucei and attempts to grow recently isolated strains of T. gambiense as cultured infective trypomastigotes in the culturing system.

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ABSTRACT

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correspond to the α-glycerophosphate oxidase activity.

Our efforts during the next year will concentrate on the identification of developmentally regulated clones of bloodstream and procyclic trypomastigotes of T. brucei, a further purification of the α-glycerophosphate oxidase system from T. brucei and attempts to grow recently isolated strains of T. gambiense in the culturing system as cultured infective trypomastigotes.

APPROACH TO THE PROBLEM

Differentiation occurs during the life cycle of African trypanosomes. It is a complex process involving the regulation of the expression of numerous genes, the expression of which is important in controlling the life cycle of these organisms. For example, in bloodstream trypanosomes, nuclear genes for the expression of the surface coat antigen are functioning. However, after differentiation to procyclic trypomastigotes, these genes no longer are expressed. In contrast, it is very clear that while no cytochromes are present in bloodstream trypomastigotes, they are essential during respiration of procyclic trypomastigotes. As we have noted previously, our approach to the development of new trypanocides is to study two specific and related areas of the molecular biology of these organisms, these areas being the expression of nuclear and mitochondrial genes. These systems are interrelated as can be seen in Figure 1.

One potential target for these parsites is the cyanide-insensitive terminal oxidase, the α-glycerophosphate oxidase system, present in bloodstream trypomastigotes. More biochemical information is needed on the properties of this unique terminal oxidase, and if one could alter the functioning of the electron transport systems in trypanosomes, perhaps it would be possible to inhibit their life cycle. In addition, in order to identify new targets for potential trypanocides, it would be extremely helpful to learn more about the properties of the mitochondria in trypanosomes, including the replication and transcription of kinetoplast DNA

and the repression and synthesis of the mitochondrial electron transport systems.

We are also interested in the inhibition of novel enzymes on processes which are under control of the nuclear genome. These include processes such as antigenic variation or synthesis of α-glycerophosphate oxidase on cytoplasmic ribosomes. Our direction in the development of new trypanocidal drugs is to gain additional knowledge on the control mechanisms involved in the transcription of nuclear and mitochondrial genes in these organisms. In this way, we hope to identify potential targets for trypanocides that so far have not been investigated. The proper establishment of the primary mode of action of a drug requires a systematic study of its effect on the various metabolic processes of the cell at the lowest concentrations that inhibit growth. Only when such a survey is complete can it be concluded confidently that a particular pathway or reaction is most sensitive to inhibition by a drug and is therefore the primary target of that drug. We are carrying out this type of systemic study with several trypanocidal drugs including berenil, suramin, antrycide and ethidium bromide.

The numerous and necessary speculative points raised in any discussion of trypanocidal drug action are now mostly capable of experimental verification with currently available techniques and data from related fields of cell and molecular biology and biochemistry. Given the necessary attention, this largely neglected but important field should yield results of considerable value, not only for an understanding both of trypanocidal drug action

and of trypanosomal metabolism, but for cell biology in general. This remains the purpose of this contract. As far as trypanocidal drug design is concerned, we strongly feel that the era of intelligent empericism is unlikely to be superseded until the balance of effort and expenditure on drug production is adjusted more favorably in the direction of research on the metabolism of trypanosomes and on the mechanisms whereby existing drugs exert their specific effects.

In developing new trypanocidal agents, we are turning our attention to investigating:

1. Application of recombinant DNA technology to a clarification of nuclear and mitochondrial gene function in trypanosomes;
2. Effects of trypanocidal drugs affecting enzyme systems in trypansomes as well as the host tissues;
3. Comparisons of homologous enzymes in host and trypanosomes;
4. Unique cell components or metabolic pathways in trypanosomes.

BACKGROUND

African trypanosomiasis is confined to Africa by the distribution of its vectors. T. gambiense infection occurs over a broad belt from Senegal in the west to the great lakes Victoria, Albert, Benguelo, Tanzania in the east, extending as far south as northern Angola. T. rhodesiense is located in east and central Africa and is scattered over a longitudinal band from the south Sudan to Mozambique. The importance of trypanosomiasis of man and animals in Africa has been summarized recently by Kershaw (1) in the statement:

> "The World Health Organization in determining the ten major health problems facing mankind places trypanosomiasis of man and his domestic animals high on the list along with malaria, cancer and heart disease."

The need for new trypanocides cannot be overemphasized. At present, chemotherapy of African trypanosomiasis is dependent on a relatively small number of synthetic drugs. Suramin and pentamidine are used for prophylaxis and treatment of early stages of the disease in man. Organic arsenicals such as tryparsamide and melaminyl compounds are used for advanced cases when trypanosomes have invaded the central nervous system. The disease in cattle and other domestic animals is controlled by quaternary ammonium trypanocides (e.g., antrycide, ethidium, prothidium, and related drugs) and by the aromatic diamidine, berenil. As pointed out recently by Newton (2), resistance has been reported

to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to another.

In human trypanosomiasis, there is still an urgent requirement for a cheap, simply administered and well-tolerated, preferably "one-shot" drug which would be as effective a prophylactic as pentamidine and active therapeutically against all stages of the infection in both Gambian and Rhodesian sleeping sickness. It should also be incapable of inducing drug resistance and active also against strains with acquired resistance to other drugs.

Possibly the two requirements of prolonged tissue retention (for prophylaxis) and ability to penetrate into the central nervous system are mutually exclusive, but with increasing knowledge of the structure and function of the so-called "blood-brain barrier," this problem should not be insuperable.

In none of the active drugs is the mode of action precisely known. An excellent review of the mode of action of trypanocidal drugs has been prepared by Williamson (3). More recent studies have suggested that berenil and ethidium bromide form complexes with DNA. In the case of ethidium, it is clear this drug is a potent and selective inhibitor of DNA synthesis. It has been shown by several investigators that both phenanthridines and acridines combine with DNA by the heterocyclic chromophore of the drug molecules becoming inserted, or intercalated, between the adjacent base pairs in the double-stranded helix of DNA. Such intercalation is achieved by a partial uncoiling of the

DNA helix which results in the base pairs above and below the bound drug molecule becoming separated by twice their normal distance (2).

More recently, it has been shown that phenanthridines also bind to supercoiled DNA of the type found in certain tumor viruses, mitochondria of many cell types and kinetoplast of trypanosomes. There is evidence that these drugs bind preferentially to such DNA *in vivo* and give rise to dyskinetoplastic trypanosomes (4) and "petite mutants" of yeast (5). The molecular basis of this preferential binding is not yet fully understood. The findings that have been observed could adequately explain the growth inhibitory activity of phenanthridine drugs but it remains to be established whether their primary action on bloodstream forms of trypanosomes is to inhibit DNA synthesis.

Berenil, an aromatic diamidine, has been shown to interact with DNA and can selectively block kinetoplast replication (6, 7). The earliest reported effect observed of berenil is the localization in the kinetoplast of *T. brucei*. This has been detected by ultraviolet microscopy within an hour of a curative dose being injected intraperitoneally into infected mice and within seconds of the drug being added to an *in vitro* suspension of trypanosomes (7). Recent work has shown that berenil can form complexes with purified DNA, but in contrast to phenanthridines, there is good evidence that the complexes are not formed by intercalation (8).

A detailed examination of kinetoplast DNA isolated from berenil-treated *T. cruzi* has shown that many of the small circular

DNA molecules appear as branched structures (6). These forms, which are thought to be replicative molecules, are rarely seen in control preparations, suggesting that berenil does not block the replication of kinetoplast DNA at initiation but binds preferentially to certain specific points in the circular DNA molecule. As for phenanthridines, it cannot be said what is the primary effect of berenil or other diamidines on trypanosomes.

The mode of action of suramin remains enigmatic even after more than a half century of use. In vitro exposure to trypanosomes to suramin at concentrations as low as 10^{-5}M is known to reduce their infectivity whereas concentrations as high as 10^{-2}M do not affect the motility or respiration of cells. As would be expected from its structure, the drug binds avidly to basic proteins and is known to inhibit many isolated enzymes (3). The most sensitive enzymes examined appear to be hyaluronidase, inhibited at 10^{-5}-10^{-6}M, fumarase, inhibited at ca. 10^{-7}M, urease at pH 5 (ca. 10^{-4}M), hexokinase (10^{-4}-10^{-5}M), and RNA polymerase (10^{-5}M) (9). Recent studies by our laboratory supported by this contract (10) and other investigations (11,12) have demonstrated that suramin also inhibits the α-glycerophosphate oxidase in bloodstream trypanosomes in vitro. Whether this is its mode of action in vitro remains to be determined.

The ready absorption of this drug by plasma proteins may well account for the long retention time of the compound in man and animals and contribute to its value as a prophylactic agent. The question of how a molecule as large as suramin enters trypanosomes is an interesting one and it seems possible that,

when protein bound, suramin actively stimulates pinocytosis. As with the other drugs that we have discussed, there is evidence that suramin becomes localized in lysosomes. Again whether this is important to the trypanocidal action of the drug or whether it is a secondary phenomenon is unknown.

The Importance of African Trypanosomiasis as a Public Health Problem.

The WHO recently published information on the importance of African trypanosomiasis as a public health problem. Human trypanosomiasis, causing sleeping sickness, and animal trypanosomiasis, referred to as nagana, affecting cattle and other domesticated animals, are the two classical notorious plagues of Africa rooted in the continent since time immemorial. Sleeping sickness constitutes a permanent and serious risk to the health and well being of at least 35 million people, and animal trypanosomiasis is the main obstacle to the development of the vast potential for livestock production in the continent. Any involvement of our military troops in areas endemic with African trypanosomiasis would be health-threatening.

Ten thousand new cases of human trypanosomiasis are known to occur yearly, but this figure does not truly reflect the importance of the disease as a public health problem. As with many tropical diseases, prevalence figures are underestimates due to failure to recognize the disease and to under-reporting. The relatively low prevalence is due to the major control efforts which have been made over the past 50 years. Without these, sleeping sickness would still be a major cause of death, as

it was at the turn of the century, with great epidemics raging along the Congo river and the northern shores of Lake Victoria costing the lives of some 750,000 people.

At the present time, some 10 million people at risk are examined annually by mobile teams at an estimated cost of 5 million dollars. Expenditure for control of tsetse flies is at least ten times higher, but the total is difficult to evaluate since most efforts are directed towards control related to animal disease. In view of the potential serious danger of sleeping sickness, national health services accord high priority to control services but efforts are frequently inadequate since sufficient resources in terms of finance, manpower and administrative facilities may not be available.

An outbreak of sleeping sickness is a dramatic event in a community since the disease causes severe symptoms due to lesions of the central nervous system and is fatal if not treated. Outbreaks may cause populations to abandon villages and fertile farmlands, and the effect is such that even after two generatins, fear of re-exposure may prevent the people from returning. Current control measures do not usually eliminate the disease; moreover, they are costly and cumbersome. With the available tools, control is a continuing effort, producing suppression rather than eradication. The experience of the past 50 years has been that whatever control efforts are interrupted, for example due to political or economic circumstances, or out of complacency, a flare-up of the disease will sooner or later occur.

A recent example was the resurgence of trypanosomiasis in Zaire in the early 1960's, when after six years of interruption of surveillance, prevalence figures rose from 0.01% to 12%, and even to 18% in some areas. Severe outbreaks are now reported from Angola, Cameroon and Sudan and particularly in the Lake Victoria region of Uganda and Kenya. Some affected villages in East Africa have been estimated to have 60-80% of the villagers infected. Recent estimates from the Ivory Coast suggest 8-10% infection with T. gambiense. It is to be expected that more outbreaks will occur in coming years unless improved control measures can be found. Development of new tools is therefore a matter of urgency, not only as a means to eradicate the disease but to provide measures which are more effective and can be more widely applied than those presently available.

RESULTS AND DISCUSSION

A. Progress in Testing Cultured-Infective Trypanosome System's Usefulness for Drug Evaluation

During this research period, we have concentrated on several areas. It has been extremely important for us to be able to grow T. rhodesiense so that we can evaluate this in vitro culturing system for its appropriateness as a system for the screening of trypanocidal drugs. We have now tested several drugs in this system including suramin, berenil, ethidium bromide, and several diamidines including pentamidine and stilbamidine in order to determine whether trypanocidal activity could be detected in this system. In all cases, these drugs were found to be effective on cultured infective trypomastigotes of T. b. rhodesiense.

In other experiments, we are now investigating the effects of allopurinol and allpurinol ribonucleoside on cultured infective trypomastigotes of T. rhodesiense. In these experiments, efforts are being made to determine whether cultured infective forms of T. rhodesiense and T. brucei can metabolize allopurinol to the aminopurinol nucleotides. The first step in these studies has been to demonstrate a biological effect of allopurinol and allopurinol ribonucleoside. The concentration range found effective for either of these compounds is 10-20 µg/ml, these concentrations inhibiting growth of the cultured infective trypomastigotes 20-30%. Thus studies have been continued with 8- (^{14}C)-allopurinol in order to determine whether this compound is metabolized by T. brucei. The radiolabeled compound was added as 20 µg/ml which has been shown to inhibit the growth of the cultured infective trypanosomes. The actively growing cultures were then allowed

to incubate for approximately one doubling time. The media was poured off and the trypomastigotes killed with cold 5% perchloric acid. The acid extracted cells were then recovered from the tissue culture flask and frozen as soon as possible. In collaboration with Dr. Randy Berens and Dr. Joseph Marr at the St. Louis University Medical Center School of Medicine, the acid-extracted cells are now being analyzed for any aminopurinol nucleotides by HPLC technique.

Efforts have been made to continue experiments on trying to grow the African trypanosomes, particularly the T. rhodesiense in the absence of the fedder layers, or tissue culture cell. Parabiotic chambers have been designed which make it possible to determine whether T. rhodesiense can be grown in the absence of tissue culture cells. The design of the flask allows the separation of the trypanosomes from the tissue culture cells in order to determine whether the growth factors can diffuse across the Millipore membrane to the trypanosomes. In several experiments, growth of trypanosomes has occurred. However, these organisms are usually procyclic trypomastigotes which have transformed from the cultured infective trypomastigotes. In several experiments, growth of the bloodstream trypomastigotes has continued. However, further experiments are required in order to determine under what conditions growth of bloodstream trypomastigotes can be continually obtained and what are the controlling growth and nutritional conditions for the success of these experiments.

Using both T. rhodesiense EATRO 1895 and T. rhodesiense

WRATat, we have demonstrated that these organisms c ndergo transformation from cultured infective trypomastigotes to procyclic trypomastigotes. In other experiments, we have found that the rate of transformation is markedly increased in the presence of 3.0mM cis-aconitate and 1.0 mM sodium pyruvate, (Figures 2A and 2B) thus demonstrating that these cultured infective trypomastigotes can continue the transformation which is an important part of the life cycle of African trypanosomes. The use of cis-aconitate was initially suggested by Dr. Reto Brun at the Swiss Tropical Institute in Basel, Switzerland. Currently, we are using this system to examine the synthesis of cytochrome oxidase during transformation from cultured infective trypomastigotes to procyclic trypomastigotes. Preliminary experiments demonstrate that the cultured infective trypomastigotes differentiate with procyclic trypomastigotes with a complete cytochrome system (Figures 2C and 2D).

Recently isolated strains of T. gambiense (ILRAD 1325 and ILRAD 1375) has been obtained from Dr. Kenneth Stuart at the Issaquah Health Research Institute in Issaquah, Washington. These strains from ILRAD in Nairobi, Kenya came from patients in Zaire and are now being used in our laboratory for this research. We have had little difficulty in growing T. b. gambiense (TTRT-1 strain) in our laboratory using several different tissue culture cell lines including embryonic bovine trachea tissue culture cells and a human fibroblast tissue culture cell line available from Flow Laboratories. Efforts are now being made to grow these recently isolated strains of T. gambiense.

B. EFFECTS OF NUCLEIC ACID INHIBITORS AND TRYPANOCIDAL DRUGS ON TRYPANOSOMES

1. Nucleic Acid Inhibitors

During transformation, T. brucei bloodstream trypomastigotes were grown hydroxyurea at concentrations ranging from 2.5 µg/ml to 200 µg/ml. The drug proved to be lethal for all concentrations except 2.5 µg/ml. Even at this concentration, the growth was slowed to 90% that of the control. Hydroxyurea is a potent inhibitor of DNA synthesis in other eukaryotes as well.

Ethidium bromide was used to inhibit mitochondrial DNA synthesis. At a concentration of 10 µg/ml, the bloodstream trypanosomes ceased cell division during transformation. However, they were unable to live in the media at this concentration for several days. At higher concentrations (25 µg/ml-100 µg/ml) the dosage proved to be lethal in 24 to 72 hours.

2. Protein Synthesis Inhibitors

Inhibitors of cytoplasmic and mitochondrial protein synthesis were added to the trypanosome media in order to characterize biochemical systems operational in differentiating bloodstream trypomastigotes as well as established procyclic trypomastigotes. Cyclohexamide (CHX) was used to inhibit cytoplasmic protein sythesis. Its action is that it blocks transpeptidation. In all concentrations tested (1 µg/ml to 100 µg/ml), the cells died in three to four days. It is known that a 1 ng/ml or less CHX has no secondary effects such as inhibition of respiration. Thus, it appears that the trypanosome cytoplasmic ribosomes are similar to cytoplasmic ribosomes found in other eukaryotes.

Inhibitors of mitochondrial protein synthesis were chlor-

amphenicol (CAP), tetracycline (TET), erythromycin (ERY) and oleandomycin (OLE). All of these drugs were tested over ranges of 50 µg/ml to 500 µg/ml in both differentiating bloodstream trypomastigotes and procyclic trypomastigotes. It has been shown that at concentratins above 250-300 µg/ml these drugs start to inhibit respiration and other cellular functins. Below this dosage level, only CAP, TET and ERY produced deleterious effects. At concentrations at 100-200 µg/ml, TET and ERY reduced cell growth from 20 to 50% that of the control. Death ensued at higher concentrations. CAP proved to be a much more potent inhibitor. At concentrations of 25-75 µg/ml, cell growth was reduced to 30-50% that of the control. At concentratins of 100 µg/ml or above the differentiating bloodstream trypomastigotes died. Growth was inhibited to 20% of the control for the procyclic trypomastigotes at 100 µg/ml. Death ensued at 200 µg/ml.

These results indicate that T. brucei has a functional mitochondrial translational system. Drug dosage levels were assayed at concentratins below those known to elicit secondary responses. Thus, either the drugs are reflecting in inhibition of mitochondrial ribosomes in T. brucei, as is seen in other eukaryotic studies, or they are affecting an entirely different area of the cell which has not been observed in other eukaryotic studies.

3. Respiratory Inhibitors

Salicylhydroxamic acid (SHAM), potassium cyanide (KCN), and Antimycin A (ANT A) were the respiratory inhibitors used to study electron transfer to molecular oxygen in T. bruc

SHAM inhibits the α-glycerophosphate oxidase which is the dominant oxidase functional in bloodstream trypomastigotes (see figure 3). KCN is an inhibitor of cytochrome oxidase, the primary oxidase in procyclic trypomastigotes. ANT A inhibits electron flow at the cytochrome $\underline{b}$-$\underline{c}_1$ complex in the cytochrome electron transport system of procyclic trypomastigotes. SHAM was administered at concentrations of 0.1mM, 0.25mM and 0.5mM. Typically, 2.2mM SHAM is administered when assaying trypanosomes polarographically. Differentiating bloodstream trypomastigotes were affected at all three concentrations. Increase in cell number was totally inhibited up to 100 hours post inoculation. After 100 hours, the cells began to divide and increase in cell number though the trypanosomes in the 0.5mM SHAM never did fully recover and maintained a relatively constant cell number.

Morphological differentiation was also slowed in the SHAM study, taking approximately 18-36 hours longer than the control. SHAM also inhibited growth in the procyclic trypomatigote study. At 0.1mM, growth was slowed to 60% that of the control. At 0.25 mM, growth was about 50% of the control, and at 0.5mM the procyclic trypomastigotes died. KCN and ANT A had no effect on differentiating bloodstream trypomastigotes at the concentrations tested (10 μM-500 μM for KCN and 0.001 μg/ml to 100 μg/ml for ANT A). KCN did inhibit cell growth in procyclic tryponastigotes at concentrations above 50 M. ANT A elicited deleterious results at concentrations of 0.001 μg/ml to 0.1 μg/ml on procyclic trypomastigotes.

The results reported here confirm previous thinking regarding

functional biochemical systems in T. brucei. The effects of SHAM on procyclic trypomastigotes was encouraging in that it adds one more piece of evidence that there is a functional SHAM sensitive oxidase present and that the procyclic trypomatigotes are to a certain degree dependent upon it.

C. Efforts to Isolate a Mitochondrial Ribosome from T. brucei

We have successfully adapted the procedure of Braly et al. (15) to the preparation of mitochondria from T. brucei. This procedure, which involves syringe lysis of hypotonically stressed cells and isopycnic banding of the lysate in a 20 to 35% renografin gradient, results in a 8-10 fold enrichment of the mitochondrial enzyme, succinate-cytochrome c reductase. From 15 liters of cultured procyclic trypomastigotes (1-2x107 cells/ml) we obtain as much as 100 mg of purified mitochondrial protein. Total RNA obtained by SDS-phenol deproteination of these mitochondria consists predominantly of two species (12S and 9S RNA; 1100 and 500 bases, respectively) when analyzed by electrophoresis through agarose containing the denaturant, methylmercury hydroxide.

During the past year, we have attempted to determine whether the two major mitochondrial RNAs are associated with a proteinaceous particle which may be a ribosome. To increase our ability to detect such particles, we label the RNAs with ^{3}H-uridine (10 Ci/ml, 1 hour, 2.5 x 10^{9} trypanosomes/ml). We have looked for ribosomes by three different strategies: (1) release of nascent peptides with puromycin, gentle lysis of the mitochondria with detergent(s), pelleting of the putative ribosomes through a sucrose pad, and characterization of the resulting pellet on sucrose gradients; (2) detergent lysis of puromycin-treated mitochondria followed immediately by centrifugating them through a sucrose gradient; and (3) isopycnic banding of lysed mitochondria in the non-ionic density medium, metrizamicide.

A variety of non-ionic detergents were surveyed for their

ability to lyse a mitochondrial suspension. Triton X-100, Triton X-100 plus deoxycholate, and N-octyl glucoside were each effective in clarifying the suspensions. Our preferred method of lysis is 1% N-octyl glycoside in 0.5 M NH_4Cl, 0.05 M $CaCl_2$ at 3.0 mg protein/ml. ($CaCl_2$ is an inhibitor of mitochondrial RNases in Neurospora).

Centrifugation of the mitochondrial lysate over 1,85 M sucrose containing 0.5 M NH_4Cl, 0.025 M $CaCl_2$, and 0.2% N-octyl glucoside results in a fairly "glassy" pellet, as expected for ribosomes. The pellet may be contaminated with membranes or the ribosomes may be extremely hydrophobic because the pellet is only sparingly soluble in a variety of low or high salt buffers containing non-ionic detergent. When resuspended, much of the pellet turns opaque white. The spectral characteristics of the soluble fraction of the pellet are suggestive of ribosomes (A_{260}/A_{280} = 1.7), but the yield of soluble material is exceedingly low (0.2 A_{260} units from the mitochondria of $8x10^{10}$ cells). We have detected 9S and 12S RNAs in this fraction on agarose-Ch_3HgOH gels, but have been unable to identify ribosomal subunits in 15 30% sucrose gradients.

Because of the tendency of the 9S and 12S RNA associated material to aggregate, we have applied mitochondrial lysates directly to sucrose gradients containing detergent. No discrete peaks were observed in the absorbance spectrum (A_{254}) or radioactivity (^3H) profile of the gradient. When individual fractions were analyzed for their RNA content, 9S and 12S species were found together throughout the gradient. We have never observed

ribosomal subunits where 9S RNA was separated from 12S RNA.

We have also attempted to circumvent the aggregation problem by banding mitochondrial lysates in 50% metrizamide. Control *E. coli* ribosomes band at ρ=1.3 in this medium after centrifugation at 110,000 x g for >48 hrs. A band also appears at this density from *T. brucei* mitochondria, but we have not detected 9S and 12S RNA in this zone, although we routinely include aurin tricarboxylic acid and PMSF to inhibit RNAses and proteases.

Given the extremely small amount of material that we are able to obtain from mitochondrial lysates, we do not believe further pursuit of this problem is warranted at the present time.

D. ONE AND TWO DIMENSIONAL ELECTROPHORESIS OF MITOCHONDRIA FROM TRYPANOSOMES

Mitochondria from both bloodstream and procyclic trypomastigotes T. rhodesiense EATRO 1895 were studied for differences and similarities of proteins present. This phase of the research utilized one and two-dimensional polyacrylamide gel electrophoresis. The use of two-dimensional electrophoresis was important due to its increased resolving power over one-dimensional gel electrophoresis. The two-dimensional system involves focusing the proteins in the first dimension, thus separating them by their various isolectric points. The focused gel was then placed in an SDS polyacrylamide gel and the focused proteins were electrophoresed into the SDS gel and separated by molecular weight.

These electrophoretic studies yielded important information regarding protein make-up of the mitochondria of procyclic and bloodstream trypomastigotes. The main differences between the two types of mitochondria is quantitative and not qualitative as was previously thought. Most of the same proteins are present in both mitochondria but in varying amounts (see figure 4). There are some differences in actual proteins present and the procyclic trypomastigote mitochondrion contains more proteins overall. This finding is not, however, surprising considering the initiation in procyclic trypomastigotes of a citric acid cycle and a cytochrome electron transport pathway.

An attempt was made to identify subunit II of cytochrome oxidase. In colloboration with Dr. Thomas Mason in the Department of Biochemistry at the University of Massachusetts, using monoclonal antibodies from the seven cytochrome oxidase subunits of yeast.

we have found similarities between subunit II of yeast and a specific protein found in mitochondria from procyclic and bloodstream trypomastigotes. Monoclonal antibody to yeast subunit II was used to look for a reacting protein in trypanosomes, presumably also subunit II. The results show conclusively that there is a specific protein in both the procyclic and bloodstream mitochondria of trypanosomes which reacts with the subunit II monoclonal antibody from yeast cytochrome oxidase (figure 5). The protein from bloodstream and procyclic trypomastigote mitochondria which reacts with the antibody is of approximately 54,000 daltons molecular weight. The reacting protein in the yeast control had a molecular weight of about 33,000 daltons. Whether this protein is subunit II of cytochrome oxidase or a closely related protein remains to be determined.

E. Identification of the Maxicircle of Trypanosoma Brucei Kinetoplast DNA as the Location of the Gene Encoding Cytochrome Oxidase Subunit II

Flagellated protozoa of the order Kinetoplastida possess an unusual extra-nuclear genome. In T. brucei, this kinetoplast DNA (kDNA) consists of a concatenated network of about 5,500 minicircles of 1 kbp each and 50 maxicircles of 20-22 kbp. We have initiated an effort to characterize gene expression in African trypanosomes using recombinant DNA technology.

Maxicircles appear to be the functional equivalent of mitochondrial DNA in other organisms. They are of appropriate size and genetic complexity to be mitochondrial DNA [16,17]. Maxicircles are homogeneous in base sequence (unlike most minicircles) and this sequence is substantially conserved between organisms of the T. brucei group [18]. Restriction maps of maxicircle DNA have been derived for three strains of T. brucei [19-21] and for a closely related species, T. equiperdum [22]. Transcripts covering most of the maxicircle have been identified in several species [23-25], whereas minicircle transcription has not been observed in Crithidia luciliae [25], T. brucei [23,24], or Leishmania tarentolae [27]. A partial minicircle transcript has been described from C. acanthocephali [28].

We utilized as a probe for COX II the mitochondrial DNA gene coding for COX II of Zea mays, which was cloned by Fox and Leaver as a 2.4 kbp EcoRI fragment into pBR322 [29]. This maize clone is denoted pZmE1. A second probe, pMT36, contains the COX II gene of Saccharomyces cerevisiae on a HaeIII fragment in pBR322 [30 and unpublished results].

The COX II insert from pZmE1 was obtained by digesting 100 µg of the plasmid DNA with EcoRI and fractionating the products on a preparative agarose gel run in 50 mM Tris borate, 1mM EDTA, pH 8.1. The insert was isolated by electroeluting it into a slot lined by dialysis tubing [31]. Insert DNA was recovered from the eluate by precipitating it with two volumes of isopropanol from 0.5 M Tris HCl, pH 8.5. The HaeIII fragment encoding COX II in pMT36 was isolated in a similar manner.

The plasmid pZmE1 used to probe kDNA digests contains the gene for COX II of maize on a 2.4 kbp EcoRI insert (Fig. 6). Southern blots were prepared from T. brucei LUMP 1026-101 kinetoplast network DNA digested with a variety of restriction endonucleases. The digestion products display characteristic profiles: minicircle fragments migrate as a broad band in the 1.0 kbp region on 0.7% agarose gels, whereas maxicircle fragments range in size from 1 kbp to about 20 kbp, depending on the restriction enzyme(s) used [20]. For each restriction profile examined, a single DNA fragment hybridized with nick-translated pZmE1 DNA at 50°C.

Digestion of kDNA with TaqI produced two fragments of approximately 2.8 kbp. (Fig. 7, lane 3). When a double digest was performed with TaqI and EcoRI, the pair of bands in the 2.8 kbp region was resolved. The larger of the two fragments does not contain an EcoRI site and hybridizes with the probe (Fig. 2, lane 4). The TaqI fragment homologous to the gene for maize COX II is also resistant to HindIII, as shown by a triple digest (Fig. 2, lane 5).

When hybridizations were performed under more stringent conditions (60°C during hybridization and washes), no signal was detected. At 50°C some hybridization of pZmE1 probe with HindIII fragments of lambda DNA was observed. These hybrids were selectively destabilized when the ionic strength of the last two filter wash solutions was lowered from 3 x SSC (Method 2) to 0.3 x SSC. Hybridization of pZmE1 with maxicircle fragments remained (data not shown).

Additional digestions of kDNA networks with HindIII (Fig. 7) and with HaeIII and EcoRI (Fig. 8) permitted the localization of the hybridizing fragment to a unique position on the physical map of the T. brucei maxicircle. Kinetoplast DNAs from procyclic trypomastigotes of T. rhodesiense EATRO 1895, T. rhodesiense WRATat 3, and T. b. gambiense TTrT-8 were digested with TaqI and EcoRI. These double digests are compared with that of T. brucei LUMP 1026-101 in Fig. 9. The maxicircle restriction sites are largely conserved between the various trypanosomes. When Southern blots of these kDNAs were probed with nick-translated pZmE1, fragments of the same size hybridized from each species.

A similar comparison of the DNA fragment profiles and hybridization patterns of T. brucei 164 IsTat 1 and T. brucei LUMP 1026-101 is shown in Fig. 10. The probe in this experiment was the EcoRI fragment of pZmE1 encoding COX II ("pZmE1 insert"), which was gel purified and labelled with [α- ^{32}P] dATP, dTTP and dCTP.

Evidence from several laboratories indicates that the maxicircle DNA is genetically active in the mitochondrion of trypanosomes. Eight RNA transcripts covering 50% of the maxicircle of T. brucei 427 have been identified. These products include the abundant putative ribosomal RNAs, as well as six species which are retained on oligo(dT)-cellulose and are presumably mitochondrial mRNAs [23]. In T. brucei 164, 12-14 transcripts covering all but one EcoRI-HindIII fragment of the maxicircle have been found [11].

We observe homology between the genes for COX II of maize and a 2.8 kbp TaqI fragment of the maxicircle. This region of the maxicircle was transcribed in both of the studies cited above [23,24]. The homology suggests that the maxicircle codes for one of the subunits of cytochrome oxidase which is made from the mitochondrial DNA of many other eucaryotic organisms. In S. cerevisiae, one of the most extensively studied systems, mitochondrial DNA codes for subunits I, II, and III of the seven subunits of cytochrome oxidase, four subunits of the oligomycin-sensitive ATPase, apocytochrome b, and one small ribosomal subunit [32].

The coding capacity of the 2.8 kbp TaqI maxicircle fragment

is more than sufficient to direct the synthesis of a protein the size of COX II (molecular weight approximately 30,000 daltons in yeast and in maize [29]. We are now in the process of cloning this region so that its identity can be definitively established by sequencing studies and codon use and the presence of intervening sequences in maxicircle DNA may be investigated.

Details of these studies including the methodology employed, can be found in the manuscript by Johnson *et al.* in the appendix (33).

F. Efforts to Solubilize and Purify the L-α-glycerophosphate oxidase

Nonionic detergents were used in our first attempts to solubilize the L-α-glycerophosphate oxidase from the membrane of crude mitochondrial fractions. Among the detergents used were Triton X-100, Nonidet P-40, Tween 20 and Tween 80. These detergents were considered because they were readily accessible in the laboratory and because they are effective in solubilizing membranes without denaturing proteins (34). Each of these detergents were used to titrate mitochondrial fractions in order to determine the maximum concentration of detergent that could be utilized without inhibiting α-glycerophosphate oxidase activity.

Crude mitochondrial fractions and renografin purified mitochondria were prepared following procedures as outlined by Braly et al. (15). L-α-glycerophosphate oxidase activity from mitochondrial fractions was measured polarographically with a Clark-type oxygen electrode. DL glycerophosphate (10mm) was added as substrate. The incubation medium was STEM (250mM sucrose, 30mM Tris, 0.5M EDTA and 1mM mercaptoethanol) buffer, pH 7.9. After a significant rate had been obtained with the addition of α-glycerophosphate, sodium deoxycholate was added to the reaction cuvette. The mitochondria were incubated in the presence of detergent for 15-20 minutes. After which the mitochondria were centrifuged at 39,000 x g for 10 minutes. The pellet was saved for determination of α-glycerophosphate oxidase activity remaining after treatment of the mitochondria with detergent. The supernatant was centrifuged at 100,000 x g for 12.2 hours in a SW-65 rotor. However, prior to this centrifugation step, an aliquot of the supernatant was

saved and assayed for α-glycerophosphate oxidase activity. After the ultracentrifugation, the pellet and the supernatant were assayed polarographically for α-glycerophosphate oxidase activity.

The procedure followed for preparing the SDS-polyacrylamide slab gel was adapted from the method of Laemmli (35). The previously mentioned nonionic detergents used to solubilize the α-glycerophosphate oxidase from mitochondrial membranes were not very useful. Concentrations of detergents less than 0.08% - 0.1 inhibited α-glycerophosphate oxidase activity. Promising results were obtained with sodium deoxycholate. Results from titration studies indicated that concentrations of sodium deoxycholate ranging from 0.02% - 0.3% did not inhibit α-glycerophosphate oxidase activity.

Mitochondrial fractions solubilized with a final concentration of 0.08% deoxycholate showed α-glycerophosphate oxidase activity when assayed with ubiquinol-1 as substrate. α-Glycerophosphate oxidase activity was present in the deoxycholate treated pellet and was also released in the supernatant (Table 1) Glycerophosphate oxidase activity was KCN insensitive and sensitive to SHAM. SHAM inhibited α-glycerophosphate oxidase activity totally in the 0.08% deoxycholate pellet. SHAM inhibited 59.6% of the α-glycerophosphate oxidase activity in the 0.08% deoxycholate supernatant (Table 1).

Results obtained from this study also indicated that upon solubilization α-glycerophosphate dehydrogenase activity was released with the α-glycerophosphate oxidase. The α-glycerophosphate

dehydrogenase activity was separate from that of α-glycerophosphate oxidase activity. In both the detergent treated pellet and the supernatant, there was no rate obtained when α-glycerophosphate was used as the substrate. This suggests a separation of the dehydrogenase component from the oxidase component. The lack of oxidation of glycerophosphate also suggested that electrons were being donated to the dehydrogenase component via α-glycerophosphate. After the α-glycerophosphate oxidase activity was inhibited by SHAM, α-glycerophosphate dehydrogenase activity could be elicited with the addition of phenazine ethosulfate as an acceptor of electrons from α-glycerophosphate dehydrogenase (Table 2).

An increase in α-glycerophosphate oxidase activity was observed to be released in the supernatant as the concentration of detergent was increased. As the α-glycerophosphate oxidase activity increased in the supernatant, the amount of activity remaining in the pellet was found to decrease as detergent concentrations increased. Glycerophoshate oxidase activity was still insensitive to KCN and sensitive to SHAM. In the 0.20% deoxycholate pellet, α-glycerophosphate oxidase activity was inhibited 73.3% by SHAM. SHAM inhibited 58.7% of the α-glycerophosphate oxidase activity in the supernatant. α-Glycerophosphate dehydrogenase activity was stimulated with the addition of phenazine ethosulfate.

Upon further centrifugation of the supernatant at 100,000 x g, a significant increase in α-glycerophosphate oxidase activity was observed. Fifty-one per cent of the total α-glycerophosphate oxidase activity present in the 39,000 x g supernatant was recovered

in the 100,000 x g supernatant and the specific enzyme activity was increased 15-20 fold.

The results obtained from the application of the solubilized pellets and supernatants on SDS-polyacrylamide slab gels are encouraging. The banding patterns are the same in the crude mitochondrial fraction and the detergent treated pellet. However, different banding patterns are obtained in the 39,000 x g rpm supernatant and the 100,000 x g supernatant. The bands in the two supernatants are the same. The different band in the supernatants suggest that different proteins were being released upon solubilization into the supernatant fractions, as compared to the proteins that remained in the pellets (Figure 11). We are currently developing a staining of procedures which will assist in identifying the enzyme activity in the gels after electrophoresis. This assay will be based on the reactivity of nitrobluetetrazolium (NBT) with superoxide which should be produced when the gel is incubated in the presence of ubiquinol-1.

CONCLUSIONS

Our primary accomplishments during this past year are:

A. Evaluated the culturing system for the sensitivity of trypanosomes against established trypanocides. Berenil and suramin were found to be extremely effective;

B. Identified a 2.8 kbp *Tag* I restriction fragment of the maxicircle as the gene for subunit II of cytochrome oxidase;

C. Solubilized the α-glycerophosphase oxidase system from *T. gambiense* using 0.08% to 0.2% deoxycholate;

D. Characterized mitochondria from procyclic and bloodstream mitochondria using one-dimensional and two-dimensional electrophoresis.

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FIGURE LEGENDS

Figure 1. The site of action of various inhibitors on the nucleo-cell sap system and the mitochondrial system.

Figure 2A. Rate of growth of transforming cultured infective bloodstream trypomastigotes of T. brucei LUMP 1026. (o——o) BSM medium alone; (•– –•) BSM + 3.0 mM cis aconitate + 1.0 mM pyruvate.

Figure 2B. Rate of growth of transforming cultured infective bloodstream trypomastigotes of T. brucei LUMP 1026. (o——o) BSM medium alone; (o–-o) BSM + 3.0 mM cis aconitate + 1.0 mM pyruvate.

Figure 2C. Absolute spectra of whole cells of T. brucei LUMP 1026 transformed from cultured infective trypomastigotes. The cells had been transformed from bloodstream trypomastigotes for 28 days and grown in the presence of BSM + 3.0 mM cis aconitate + 1.0 mM pyruvate. (——) The cells were reduced endogenously and run against a solution of antifoam of comparable density; Full scale: 400nm - 500nm-1.0A; 500nm - 650nm-0.2A; (---) The cells were reduced with dithionite and run against a solution of antifoam of comparable density; Full scale: 400nm - 500 nm-2.0A; 500nm - 650nm-0.2A. Cell concentration of 8.0×10^8 cells/ml.

Figure 2D. Difference spectra of whole cells of T. brucei

LUMP 1026 transformed from cultured infective trypomastigotes. The conditions were the same as Figure 2C except the samples were bubbled with CO and run as difference spectra. The full scale of the difference spectrum using dithionite was 0.2A from 400 - 500nm. From 500 - 600nm and in the entire spectrum where the cells were reduced endogenously, the full scale was 0.1A.

Figure 3. Effect of salicylhydroxamic acid (SHAM) on differentiating bloodstream trypomastigotes of T. brucei. (o---o) control; (□---□) 0.1mM SHAM; (●—●) 0.25 mM SHAM; (▲---▲) 0.5mM SHAM.

Figure 4. Outlined regions of similarity of proteins between bloodstream and procyclic trypomastigotes T. brucei run on two-dimensional Gels (A). Procyclic trypomastigote mitochondria; (B) Bloodstream trypomastigote mitochondria.

Figure 5. Monoclonal antibody to subunit II of cytochrome oxidase - Verification of the subunit in yeast as well as bloodstream and procyclic trypomastigotes of T. brucei (Lane 1): Molecular weight standards (transferin, 90K; BSA, 68K; ovalbumin, 43K; catalase, 30K; and lysozyme, 14K). (Lane 2) yeast mitochondria. (Lane 3) procyclic trypomastigote mitochondria; (Lane 4) bloodstream trypomastigote mitochondria. Autoradiograph of proteins reacting with the monoclonal (COX II) antibody are to

the right in lanes 2, 3 and 4.

Figure 6. Plasmid containing the gene for cytochrome oxidase subunit II of maize (pZmE1). (Lane 1) Lambda DNA digested with HindIII. (Lane 2) pZmE1. (Lane 3) pZmE1 digested with EcoRI.

Figure 7. Hybridization of pZmE1 to Southern blots of T. b. brucei LUMP 1026 kDNA digested with several enzymes. The gel was 0.7% agarose; hybridization was by Method 1. (Lane 1) Lambda DNA digested with HindIII. (Lane 2) kDNA digested with HindIII. (Lane 3) kDNA digested with TaqI. (Lane 4) kDNA digested with TaqI and EcoRI, (Lane 5) kDNA digested with TaqI, EcoRI, and HindIII.

Figure 8. Hybridization of pZmE1 to Southern blots of T. b. brucei LUMP 1026 kDNA digested with HaeIII and EcoRI. The gel was 1% agarose; hybridization was by Method 1. (Lane 1) Lambda DNA digested with HindIII, (Lane 2) kDNA digested with HaeIII. (Lane 3) kDNA digested with EcoRI.

Figure 9. Hybridization of pZmE1 to Southern blots of digested kDNA from four brucei group trypanosomes. Each kDNA sample was digested with TaqI and EcoRI. The gel was 1% agarose; hybridization was by Method 2. (Lane 1) Lambda DNA digested with HindIII. (lanes 2 & 6) T. b. gambiense TTrT, (lanes 3 & 7) T. b. rhodesiense WRATat, (Lanes 4 & 8) T. b. rhodesiense EATRO 1895, (Lanes 5 & 9) T. b. brucei LUMP 1026-101.

Figure 10. Hybridization of"pZmEl insert" to Southern blots of kDNA from T. b. brucei LUMP 1026-101 and T. b. brucei 164 Istat 1. Each kDNA sample was digested with TaqI, HindIII and EcoRI. The gel was 0.7% agarose; hybrization was by Method 2. (Lane 1) Lambda DNA digested with HindIII, (Lane 2) T. b. brucei LUMP 1026-101, (Lane 3) T. b. brucei 164 Istat 1.

Figure 11. Electrophoresis of mitochondrial preparation from T. gambiense in SDS-polyacrylamide slab gel. (Lanes 1 and 8) crude mitochondrial preparation; (Lanes 2 and 5) mitochondrial pellet from 39,000 x g centrifugation; (Lanes 3 and 6) supernatant from 39,000 x g centrifugation (Lanes 4 and 7) supernatant from 100,000 x g centrifugation.

Table 1: SHAM Sensitivity of α-Glycerophosphate Oxidase Activity After Solubilization With Deoxycholate.

	Control	0.08% Deoxycholate		0.20% Deoxycholate	
		Pellet	Supernatant	Pellet	Supernatant
SHAM Sensitivity α-GP	94.1%	--	--	--	--
Ubiquinol-1	100.0%	100.0%	59.6%	73.3%	58.7%

45

Table 2: Solubilization of α-Glycerophosphate Oxidase from Trypanosoma gambiense with Deoxycholate.

	Control	0.08% Deoxycholate		0.20% Deoxycholate	
		Pellet	Supernatant	Pellet	Supernatant
Specific Activity: (ng atoms O_2/min/mg protein)					
α-GP	213.8	--	--	--	--
Ubiquinol-1	656.4	1350.0	3275.0	300.0	6060.0
Stimulation with Phenazine Ethosulfate (ng atoms O_2/min/mg protein)	--	12820.0	15640.0	1809.1	20210.0

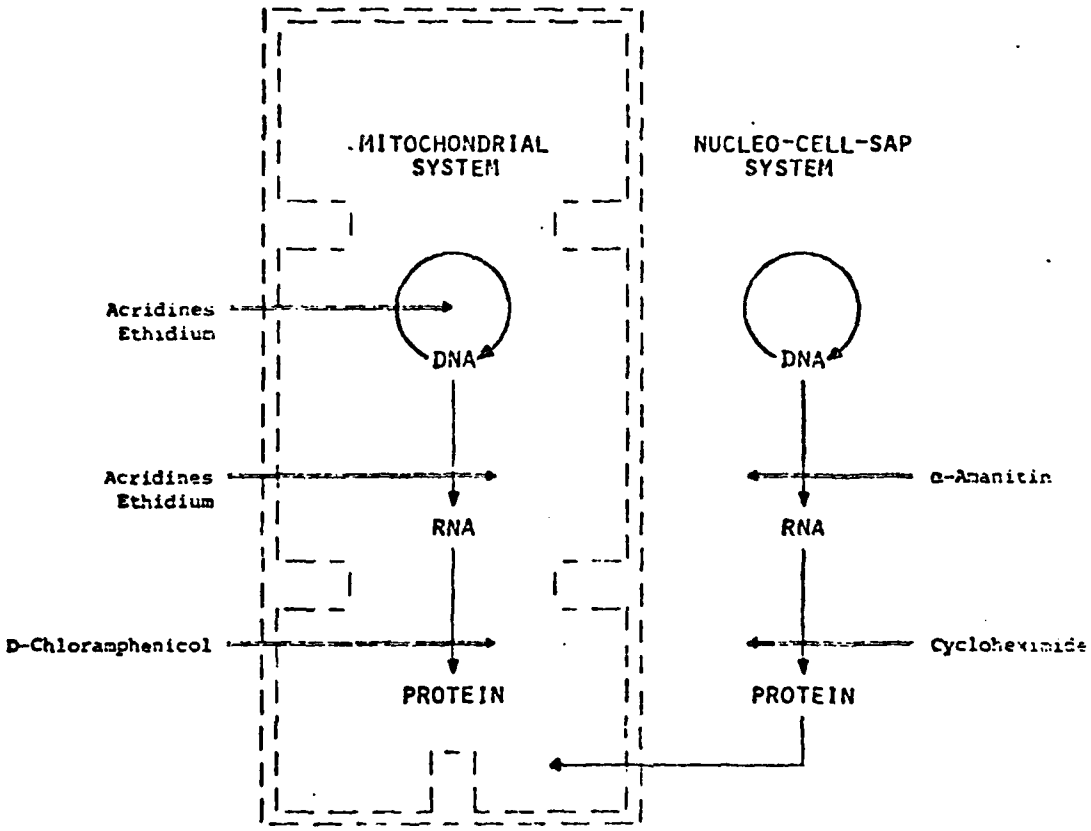

MITOCHONDRIAL SYSTEM
NUCLEO-CELL-SAP SYSTEM
Acridines Ethidium
DNA
DNA
Acridines Ethidium
RNA
RNA
α-Amanitin
D-Chloramphenicol
PROTEIN
PROTEIN
Cycloheximide

Figure 1

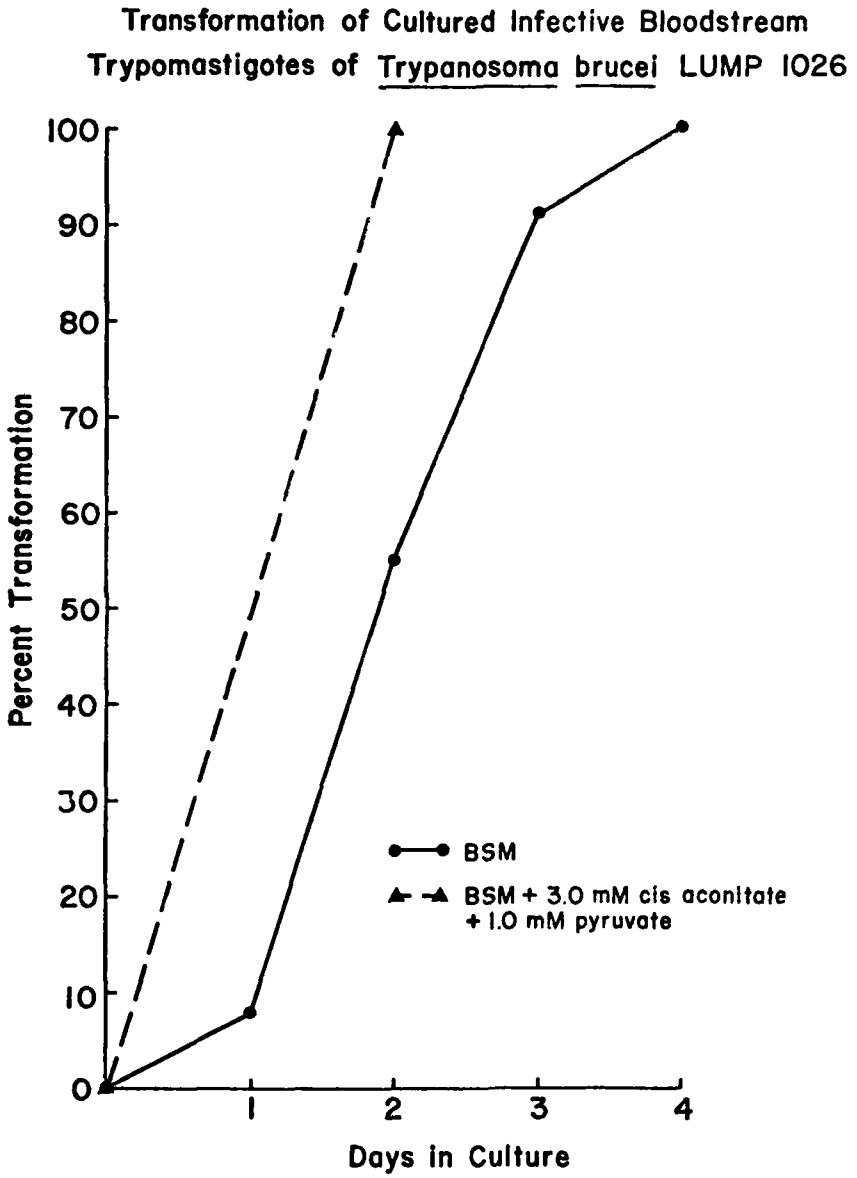

Transformation of Cultured Infective Bloodstream
Trypomastigotes of Trypanosoma brucei LUMP 1026
Percent Transformation
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
Days in Culture
BSM
BSM + 3.0 mM cis aconitate
+ 1.0 mM pyruvate

Figure 2A

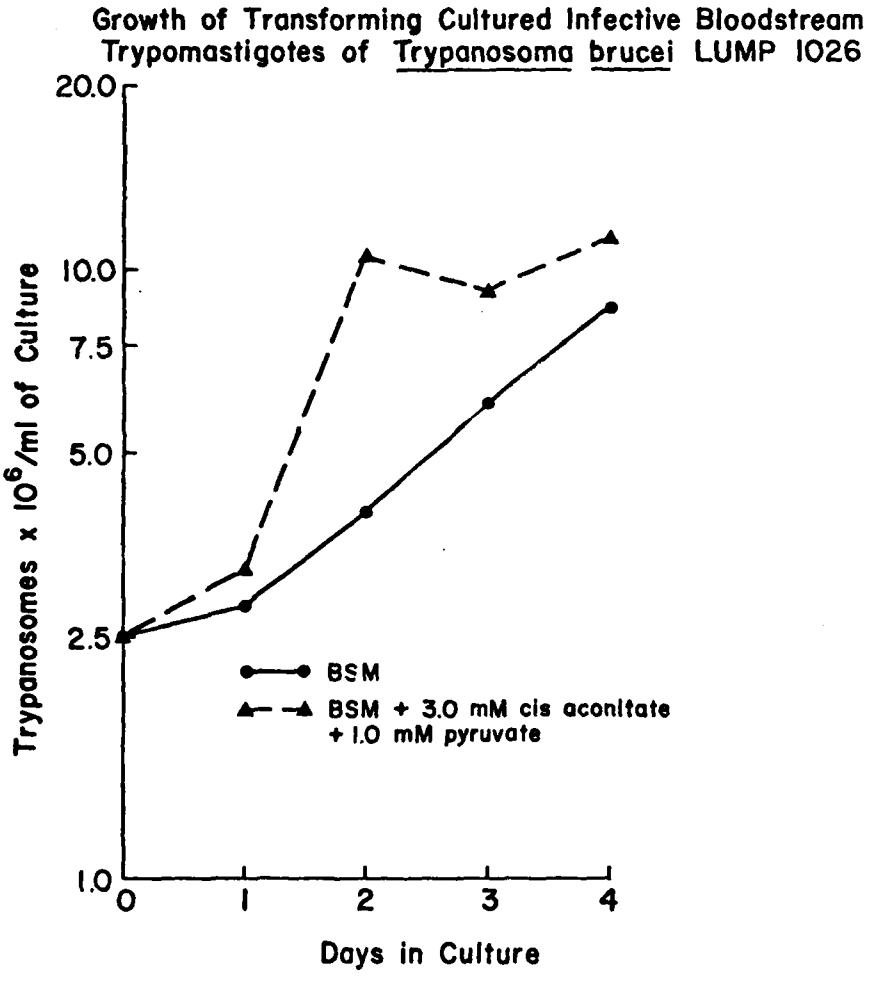

Growth of Transforming Cultured Infective Bloodstream Trypomastigotes of Trypanosoma brucei LUMP 1026
20.0
10.0
7.5
5.0
2.5
1.0
Trypanosomes x 10^6/ml of Culture
0
1
2
3
4
Days in Culture
BSM
BSM + 3.0 mM cis aconitate
+ 1.0 mM pyruvate

Figure 2B

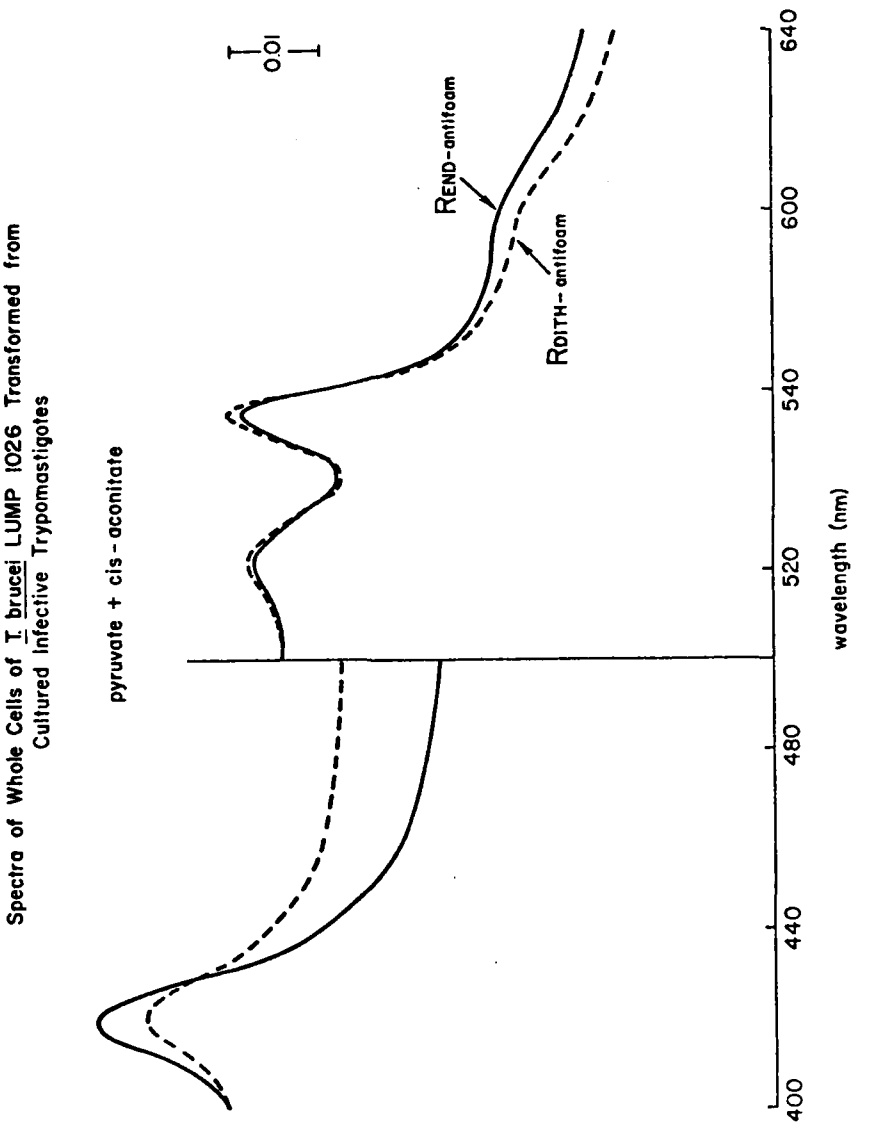

Spectra of Whole Cells of T. brucei LUMP 1026 Transformed from
Cultured Infective Trypomastigotes
pyruvate + cis-aconitate
0.01
REND-antifoam
ROITH-antifoam
400
440
480
520
540
600
640
wavelength (nm)

Figure 2C

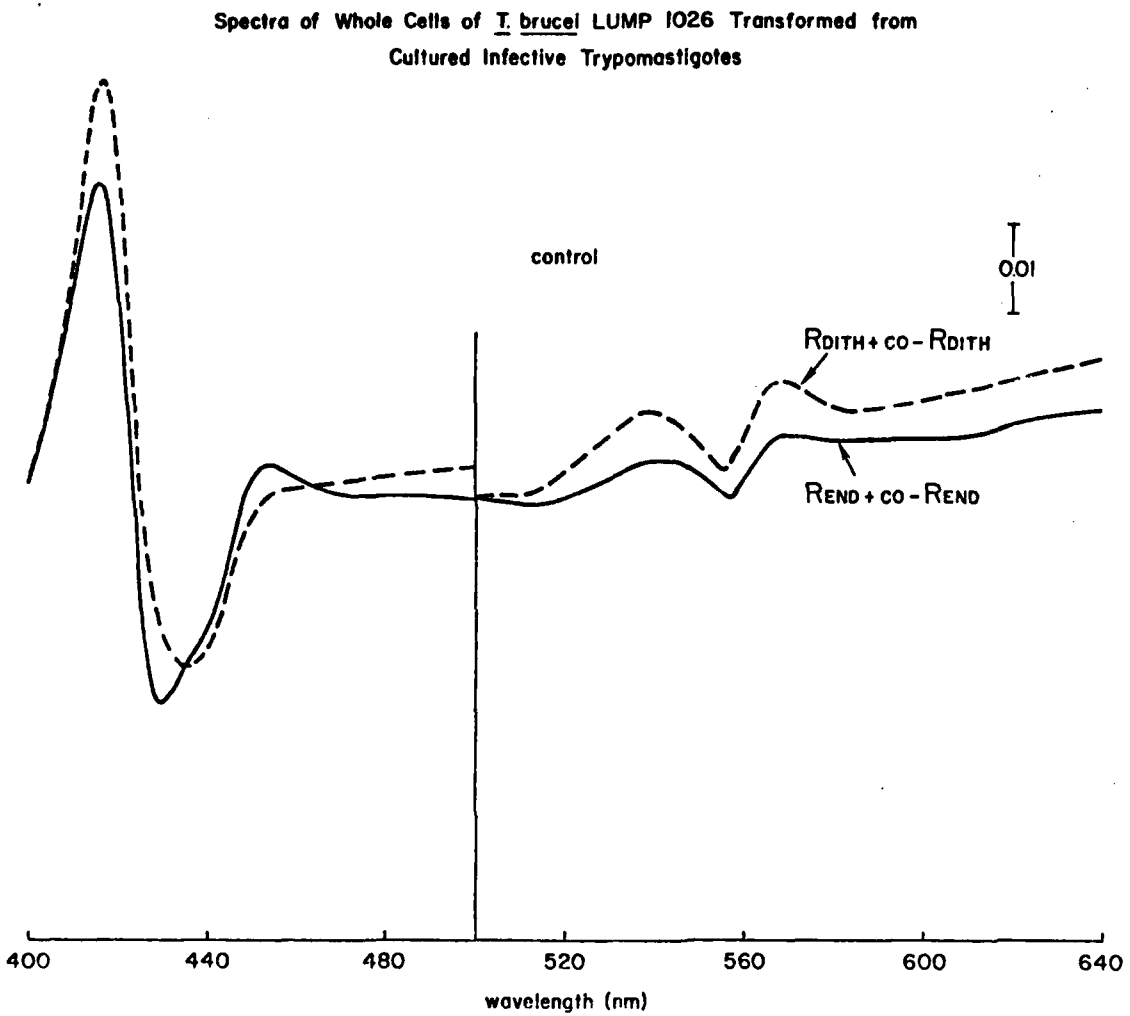

Spectra of Whole Cells of T. brucei LUMP 1026 Transformed from
Cultured Infective Trypomastigotes
control
0.01
RDITH + CO - RDITH
REND + CO - REND
400
440
480
520
560
600
640
wavelength (nm)

Figure 2D

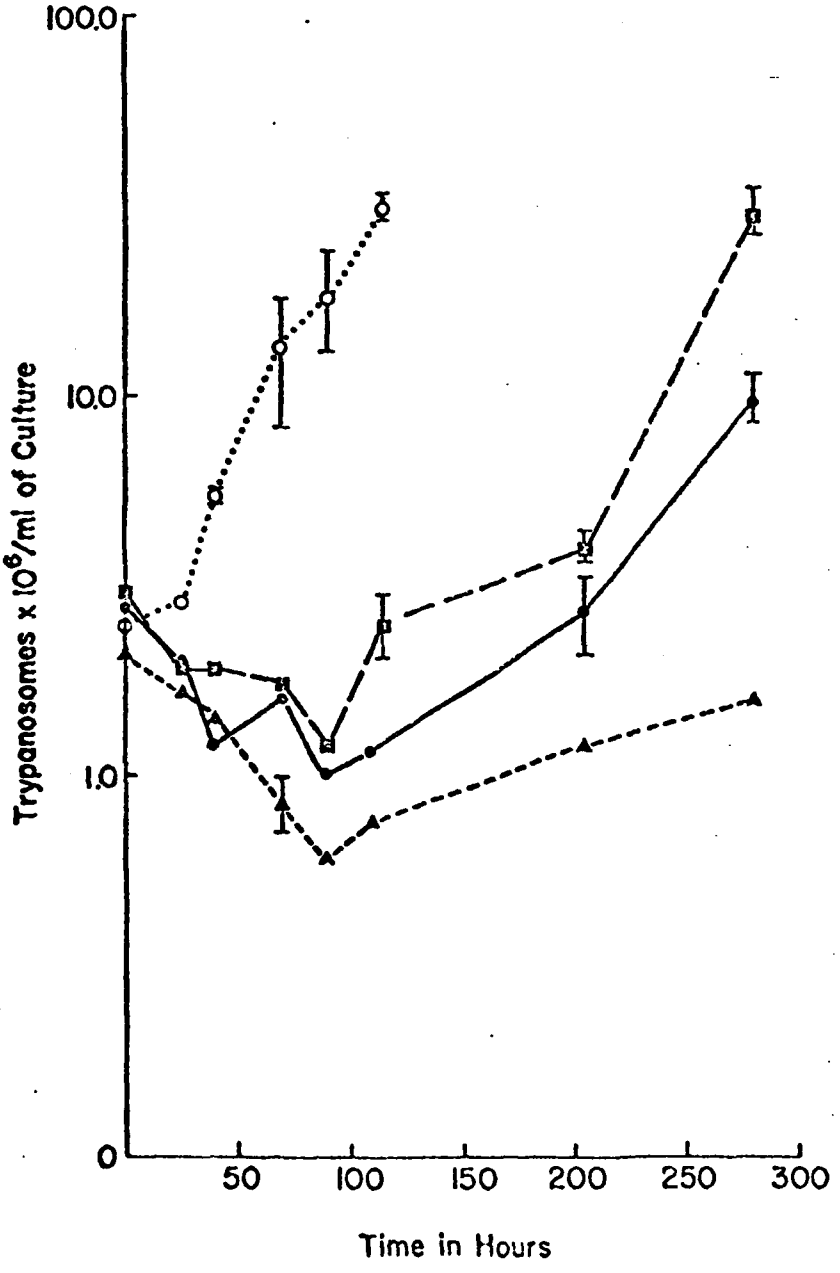
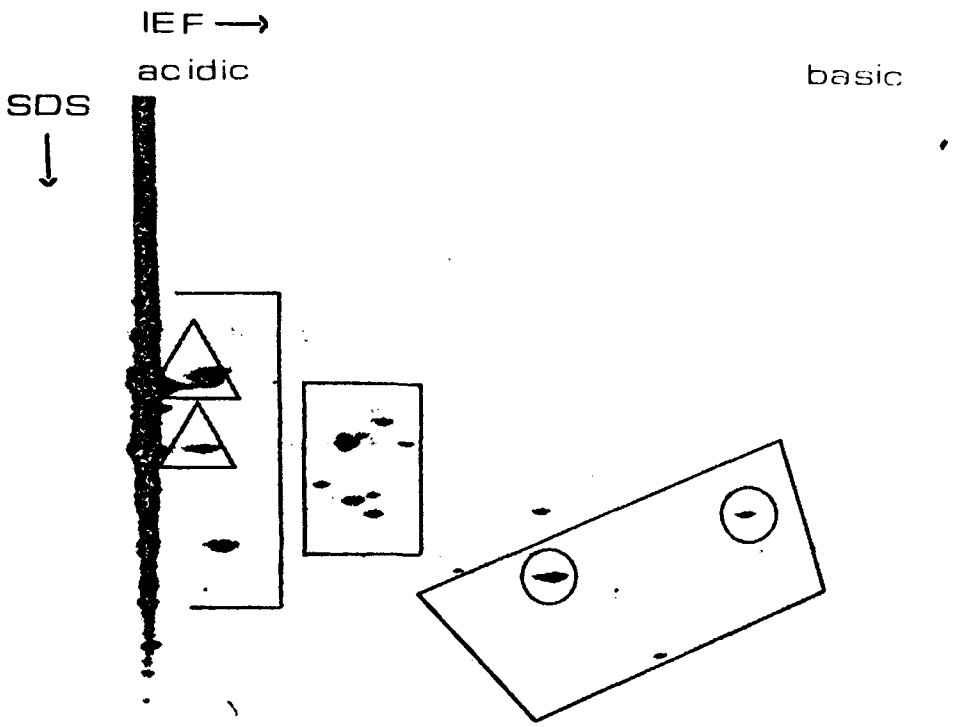
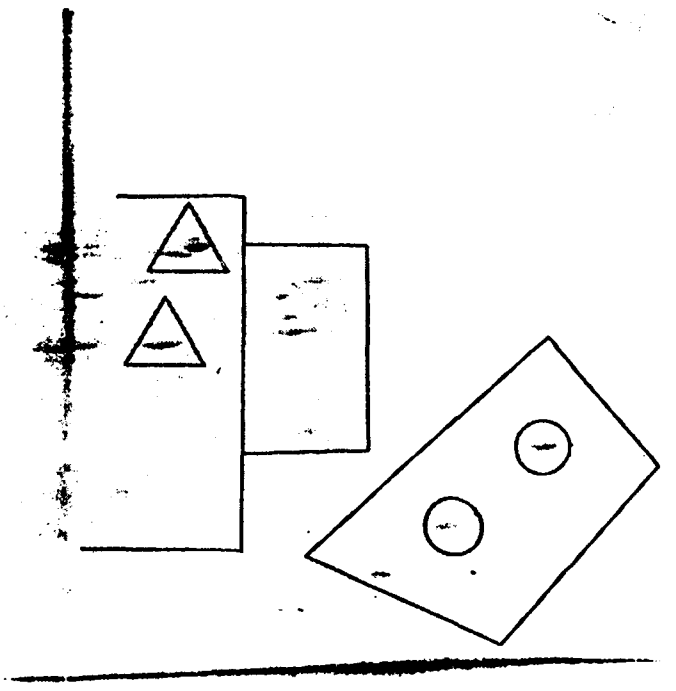

100.0
10.0
1.0
0
Trypanosomes x 10⁶/ml of Culture
50
100
150
200
250
300
Time in Hours

Figure 3



A



B

Figure 4

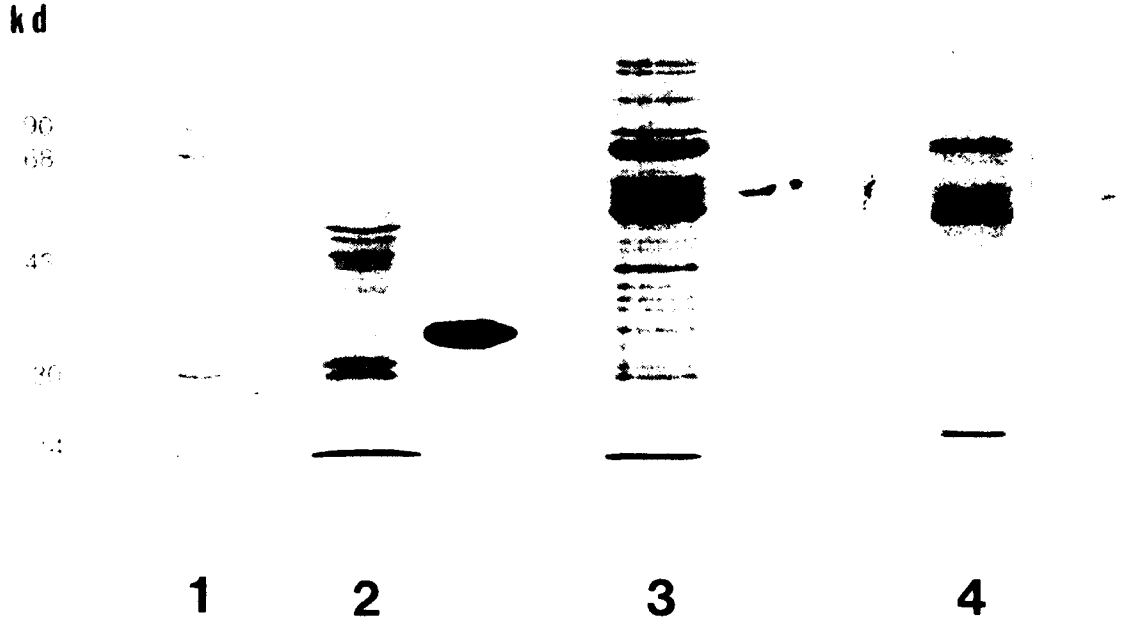


Figure 5

Figure 6

Figure 7

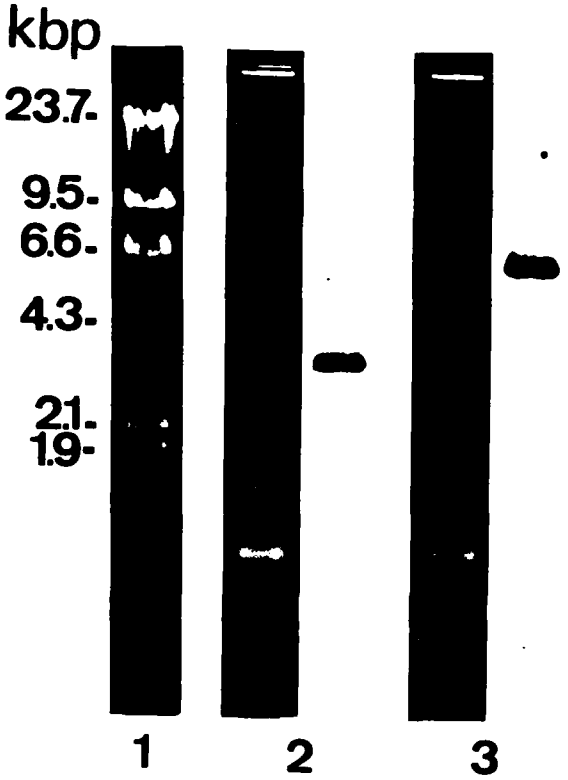


Figure 8

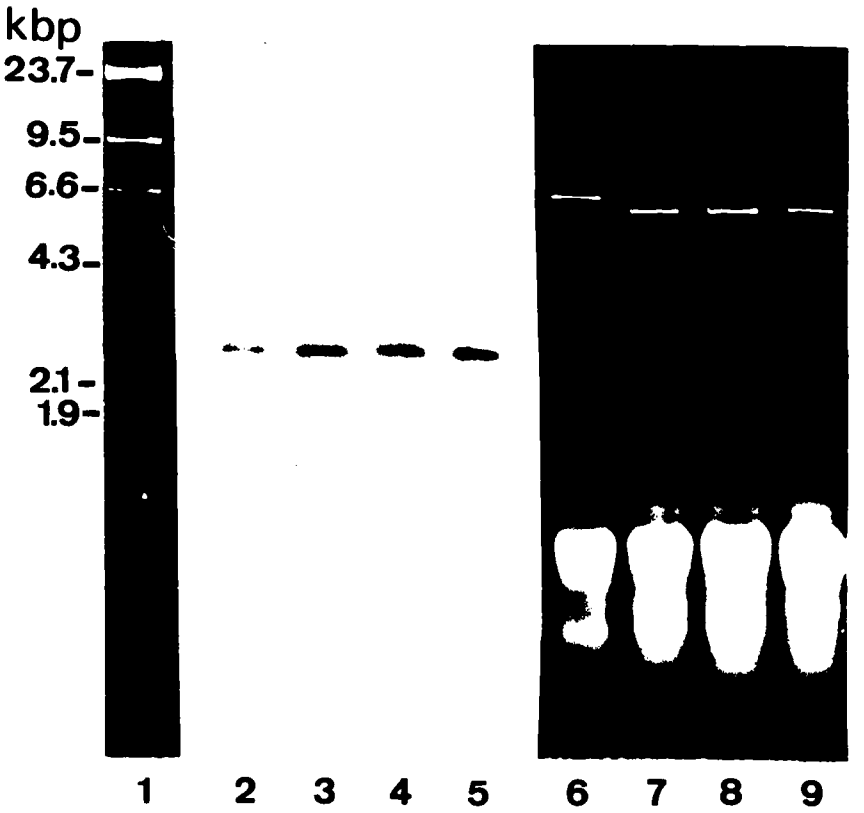


Figure 9

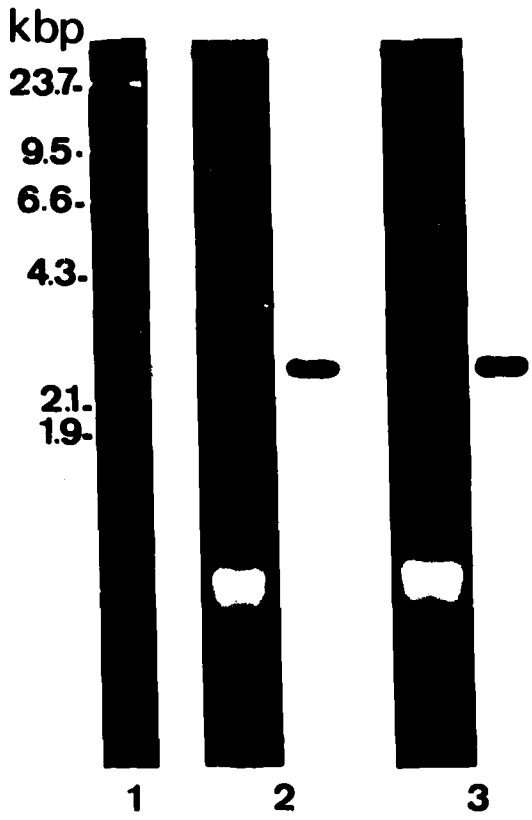


Figure 10

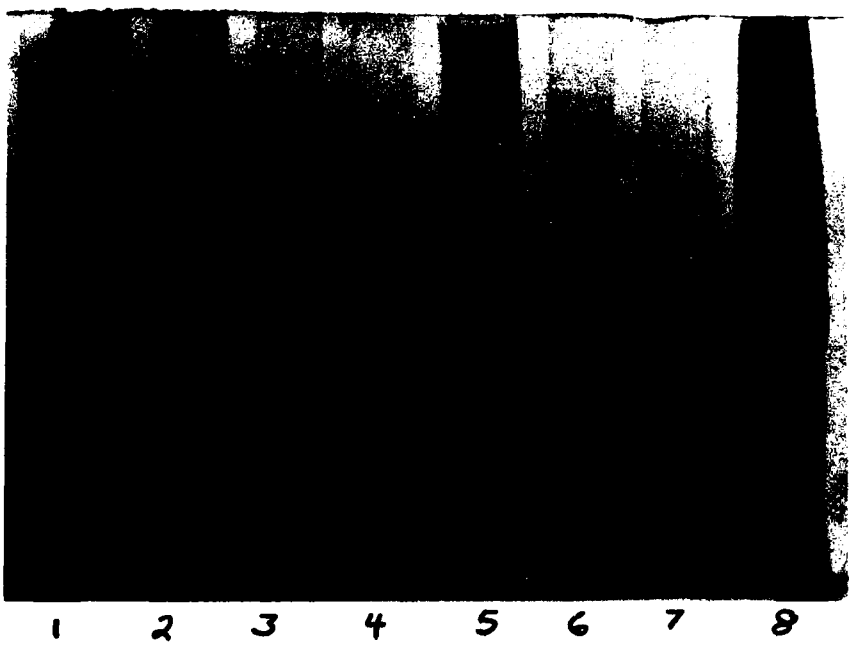
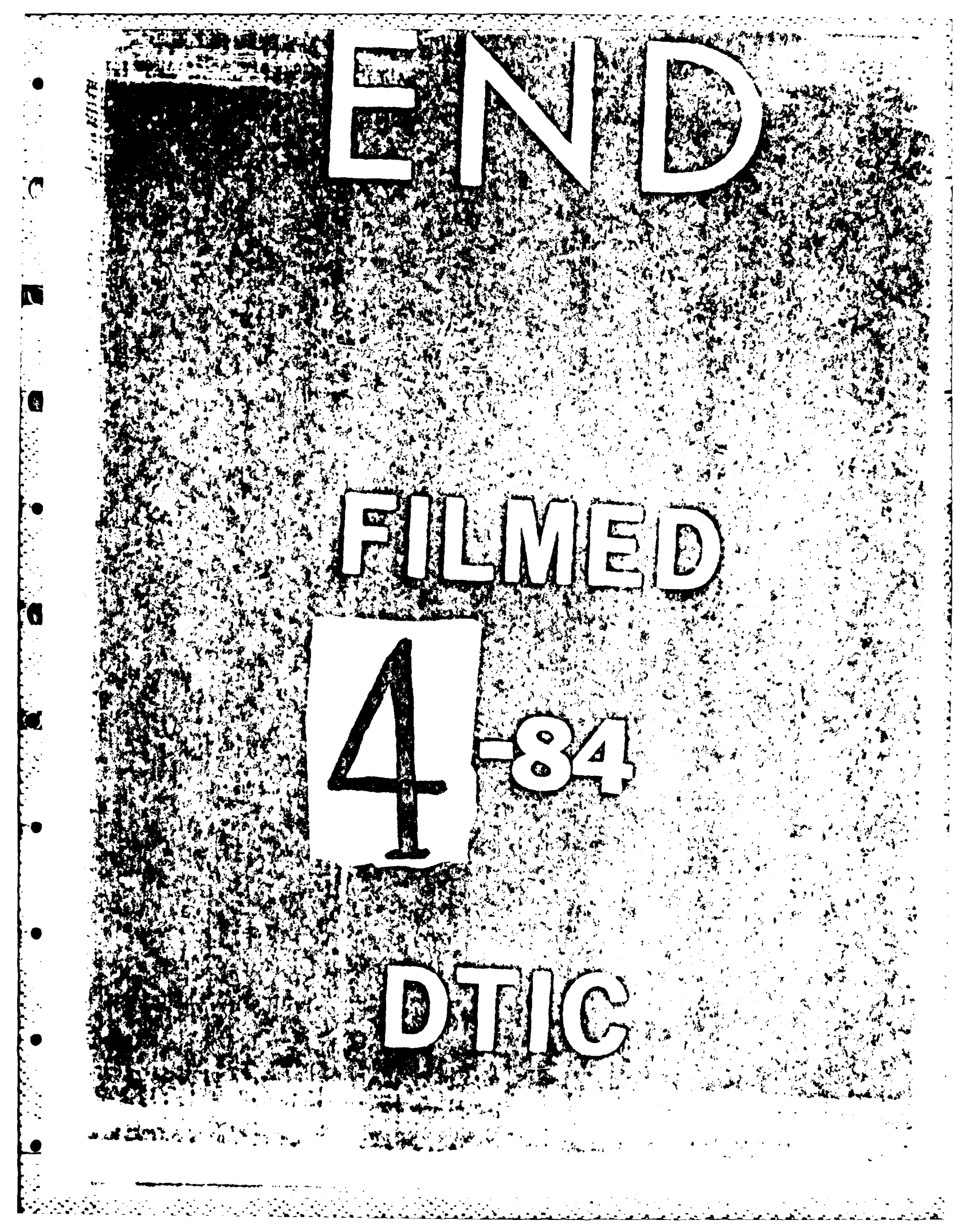


Figure 11


END
FILMED
4-84
DTIC